Comprehensive profiling of immune responses in MARV survivors demonstrates robust Th1-skewing with short-lived neutralizing antibody responses.

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Introduction

The genera *Marburgvirus* and *Ebolavirus* comprise the family *Filoviridae*, which contains the etiological agents that cause Marburg virus disease and Ebola virus disease, respectively. Ebola virus (EBOV) recently caused an outbreak of truly unprecedented scale, spanning two years and infecting more than 28,000 individuals. Previously, the largest filovirus outbreaks had numbered in the tens to hundreds. While EBOV is responsible for the greatest number of human filovirus infections, Marburg virus (MARV), Sudan virus (SUDV), and Bundibugyo virus (BDBV) have all caused outbreaks that numbered over 100 cases – twice, in the cases of MARV and SUDV. EBOV may garner much of the attention, but it is only one of several filoviruses that could potentially kindle outbreaks of massive scale. To date, there have been 11 incidences of MARV transmission to the human population. While many outbreaks have been of limited scale, afflicting fewer than 5 individuals per instance, two outbreaks alone account for 406 cases of MARV infections that had fatality rates of 83% and 90% (Bausch et al., 2006; Towner et al., 2006). A precedent already exists for the importation of MARV to other countries from travelers that visited MARV hotspots (Centers for Disease and Prevention, 2009; van Paassen et al., 2012), which was a massive concern during the 2013-2015 EBOV outbreak in West Africa. Simple luck or coincidence prevented these MARV outbreaks from growing into larger problems.

In 2012 alone, four distinct filovirus outbreaks occurred in Africa. Three outbreaks occurred in Uganda (two of SUDV, one of MARV), with the fourth occurring in the Democratic Republic of Congo (BDBV) (Albarino et al., 2013). Sequencing of viral genomes isolated from two fatal cases of MARV in 2012 indicated a high similarity with the original MARV isolate (Albarino et al., 2013; Amman et al., 2012). MARV case fatality rates (CFRs) vary from 23-90% in cases where more than one individual was infected. The CFR for the MARV outbreak in 2012 was 27%, in keeping with the outbreak that was caused by the MARV isolate bearing the greatest homology to the 2012 isolates (original MARV isolate in 1967; CFR 23%) (Albarino et al., 2013; Amman et al., 2012; Bukreyev et al., 1995; Slenczka and Klenk, 2007).

Profiles of immunity developed in EBOV survivors have begun to shed light on immune responses that had been understudied. EBOV survivors treated in the US, either after exposure in West Africa or after nosocomial infection in the US, developed IFNγ- and TNFα-positive CD4 and CD8 T cell responses to various EBOV proteins (McElroy et al.,

2015). This was the first time antigen-specific responses in T cells had been directly demonstrated in human survivors of filovirus infection, whereas antibody responses had been characterized previously (Baize et al., 1999; Gupta et al., 2012; Ksiazek et al., 1999; Sobarzo et al., 2012). Prior to this, some of the best information to date came from mRNA analysis of PBMCs collected during an Ebola virus outbreak in Gabon, which revealed evidence for T cell responses in both fatal and non-fatal cases. During the symptomatic phase of the infection, both fatal and non-fatal cases appeared largely indistinguishable based on the expression of similar cytokine transcripts. Convalescent cases, however, had very clear increased transcript levels for effector molecules such as FasL and perforin. This observation is suggestive of CD8 T cell responses and indeed, transcripts levels for CD8 were increased as well (Baize et al., 1999). Additionally, total CD8 T cell counts were also found to be increased in SUDV infections (Sanchez et al., 2004). IL-2, IFNγ and TNFα were also found to be present in serum samples from survivors and non-survivors of an EBOV outbreak in DRC (Villinger et al., 1999). These studies demonstrate that adaptive immune responses do occur in infected individuals with both fatal and non-fatal outcomes. Abortive antibody responses (low virus-specific IgM production, no IgG) were observed among fatal cases, in contrast to non-fatal cases where IgM responses matured into an IgG response. While these studies contributed to the field's understanding of early filovirus immune responses, modern techniques and assays may further our understanding of which responses predict favorable outcomes, and possibly tease apart differences in immune responses to various filovirus family members.

Beyond IgG or IgM ELISAs performed for diagnostic purposes, virtually the entirety of the literature available regarding filovirus immune responses in humans comes from studies of survivors of EBOV, SUDV, and BDBV. At the present time, it is unknown if immune responses from MARV survivors will present similar profiles to those described for Ebola viruses, or if there will be critical differences. In these studies we provide, to our knowledge, the first comprehensive immune profiling of responses in human MARV survivors. Identification of a consensus immune response elicited by filovirus infection would be highly valuable for the evaluation of candidate vaccines that would seek to be broadly protective.

Results and Discussion

T cell cytokine responses.

Heparinized blood samples from the Uganda 2012 MARV outbreak survivors, as well as local individuals who had not been infected, were obtained approximately 9 months after the resolution of the outbreak. Health questionnaires at the time of phlebotomy indicated that all subjects were relatively healthy and not experiencing any illness. Our use of whole blood cultures to measure filovirus survivor immune responses has been described previously (Sobarzo et al., 2016). Briefly, 0.25mL fresh whole blood was mixed with 0.75mL RPMI-5% FBS and cultures were incubated with MARV or SUDV glycoproteins (GP) or noninfectious irradiated whole virus preparations of MARV or SUDV. In these studies, cultures were supplemented after 18hrs with brefeldin A and incubated for a further 4hrs. After 22hrs total, cultures were vortexed, cells were pelleted, and the resultant supernatant was collected. Cells were subjected to two 5-minute incubations with a Tris-ammonium chloride solution to lyse red blood cells and subsequently stained for surface and intracellular antigens. This approach enabled a flow cytometry analysis of cytokine response with matched supernatants for an expanded cytokine analysis by ELISA.

A representative set of flow plots for CD4 and CD8 T cell responses from a MARV survivor is shown in figure 1A. Plots are gated sequentially on lymphocytes, singlets, live cells, and CD3+ CD4+ or CD8+ events. Resting, SUDV GP, MARV GP, and irradiated SUDV cultures elicited minimal to no cytokine secretion from either CD4 or CD8 T cells. On the other hand, irradiated MARV stimulation elicited robust cytokine expression in survivor CD4 T cells (upper panels), comprised of IFNγ and IL-2 double- and single-positive responses. Survivor CD8 T cells (lower panel) displayed a more limited response, consisting almost exclusively of IFNγ production. Cells from uninfected control individuals showed no reactivity to viral antigens (data not shown).

Individual survivors' CD4 T cell responses are depicted in figure 1B. IFNγ and IL-2 single- or double-positive responses to irradiated MARV stimulation are shown for each survivor. As only the irradiated MARV stimulation resulted in cytokine production, we focused further on that response to determine its composition. Resting values were subtracted to account for nonspecific responses. All but one survivor (S1) had IFNγ single-positive responses, whereas all survivors had IFNγ, IL-2 double-positive and IL-2 single-positive responses. The magnitude of each

Comment [II1]: Were these protein specific or peptide specific – Big difference. I think Sobarzo was the first paper to whole proteins.

Comment [(2]: These were protein-specific but elicited with peptide pools. No one else has used whole protein, I believe.

Comment [II3]: I thought the first BDBV outbreak was in 2007. This ref is from 2004! This reference is for Sudan.

Comment [(4]: Corrected – thanks!

individual's response varied greatly, with some survivors having barely detectable responses (S1, S6), whereas others were very robust (S2, S5) (figure 1B). Levels of CD8 T cell responses were low and did not permit a similar analysis (figure 1A and data not shown).

Secreted cytokine analysis

To complement the flow cytometry analysis, we performed a multiplex ELISA assay with the culture supernatants to analyze a broader range of cytokines. We focused on five cytokines that are germane to adaptive immune responses: IL-2, IFNγ, TNFα, IL-4, and IL-5. Average resting and irradiated SUDV-stimulated expression levels of each cytokine were low for uninfected control and survivor samples. MARV stimulation elicited IL-2, IFNγ and TNFα expression that was significantly higher than resting and SUDV-stimulated cultures ($p < 0.05$). IL-4 was not measured in any of the cultures and only a slight, insignificant increase in IL-5 expression was measured following irradiated MARV stimulation.

CD40L expression and cytokine responses

Whereas we demonstrated the utility of whole blood cultures to measure T cell responses in MARV survivors (figure 1), the use of PBMCs allows for the analysis of greater cell numbers. Additionally, purified PBMCs can better elaborate cytokine responses in comparison to whole blood cultures (Hoffmeister et al., 2003). We therefore developed an assay to use PBMCs instead of whole blood to attempt a more detailed and robust analysis of T cell responses in MARV survivors. PBMCs were cultured with the specific antigens as before, with the exception that monensin (instead of brefeldin A) was added after 2hr of culture to begin trapping intracellular cytokines. Additionally, fluorescently-labeled antibodies against CD107a and CD40L were added at this time to enable detection of degranulation (Betts et al., 2003) and CD4 T cell activation (Chattopadhyay et al., 2006), respectively. Cultures were then incubated for a further 16hr. Cell viability gated on lymphocytes after culture (18hr total, 16hr with monensin) was measured by an amine-reactive dye and found to be 91 ± 5.8% inclusive of all survivors and culture conditions (data not shown).

With a combination of CD40L and IFNγ expression, we identified activated CD4+ T cells after stimulation with irradiated MARV antigen. IFNγ expression was coordinately expressed with CD40L, which is consistent with the description of CD40L expression as an activation marker (figure 3a, upper panel) (Chattopadhyay et al., 2006). TNFα and IL-2 expression also followed the same pattern with regards to CD40L expression (data not shown). PBMCs from uninfected controls demonstrated no cytokine response to irradiated MARV antigen (figure 3a, middle panel). Representative IFNγ and TNFα staining, gated on CD40L+ CD4+ T cells, is depicted in figure 3a (lower panel) for all survivors after irradiated MARV antigen stimulation.

To measure the overall magnitude of the CD4+ T cell response, we used a Boolean gating strategy to determine the frequency of IL-2+ or IFNγ+ or TNFα+ events that were identified by CD40L expression after stimulation (figure 3b). CD40L expression has been shown to increase in a nonspecific manner during culture (Chattopadhyay et al., 2006) and this analysis enabled us to determine the frequency of only cytokine-expressing, CD40L+ CD4 T cells to give a more accurate representation of the specific response. Resting, SUDV GP, and irradiated SUDV antigen cultures elicited negligible CD40L+ cytokine+ responses (figure 3b), reinforcing both the utility and specificity of the stimulation and analysis. MARV GP elicited responses of very low magnitude, whereas the CD40L+ cytokine+ response to irradiated MARV was more robust in comparison (figure 3b). The composition of each individual survivor's CD40L+ response to MARV stimulation in terms of IFNγ, TNFα, and IL-2 is depicted in figure 3c. Resting culture values were subtracted to account for any background cytokine expression. The most dominant response was found to be cells producing IFNγ, TNFα and IL-2, whereas the double- and single-positive responses for these cytokines varied to a greater extent amongst survivors (figure 3c).

We had measured CD8 T cell responses in whole blood cultures previously (figure 1 and data not shown), but the IFNγ and IL-2 cytokine responses were very low in magnitude. We considered it possible that perhaps these parameters were not capturing the totality of the CD8 T cell response. We incorporated CD107a staining into our analysis to detect any CD8 T cells that had degranulated and/or produced cytokines in response to MARV antigens. To this end, we analyzed CD107a and IFNγ expression after MARV stimulation. Control CD8 T cells demonstrated

no IFNγ expression in response to MARV stimulation, whereas CD107a expression was found in both resting and MARV-stimulated cultures. This apparently nonspecific CD107a expression was found in resting cultures for survivors' CD8 T cells as well. Only CD107a expression in the context of IFNγ demonstrated specificity with regards to MARV stimulation, and only in a subset of survivors (figure 4, right panels and data not shown). These results confirm our earlier finding with CD8 T cell responses in MARV survivors, and add the additional functional characteristic of apparent cytotoxic responses.

Antiviral antibody responses

To address the humoral immune response to MARV infection, we collected serum from uninfected control donors and survivors and analyzed these samples for IgG antibodies against irradiated MARV antigen. Serum samples were serially diluted to determine an end titer. All survivors had IgG responses to irradiated MARV, reaching an end titer between 4.25 and 6 (LOG$_{10}$ serum dilutions) (figure 5a). Cell lysates expressing various MARV proteins were employed to determine the individual protein specificity of the MARV IgG response. Responses to lysate-derived antigens were denoted as +/- based on a signal-to-noise ratio of cell lysates without MARV proteins (table 1). All survivors were found to have IgG responses against MARV NP and GP, but not against VP35 or VP24 (table 1). Survivors 2-7 had IgG responses to VP40 and survivors 1-4 and 5-6 had responses to VP30. Control sera were also included in these analyses and found to be nonreactive against lysates bearing MARV proteins (data not shown).

In order to address one potential function of the MARV-specific serum antibodies, we used a plaque reduction/neutralization test (PRNT) to determine if survivor serum could neutralize virus *in vitro*. Serum samples were serially diluted beginning at 1:10 and pre-incubated with MARV. This mixture was then used to inoculate Vero E6 cells and resulting plaques were counted. Serum from only two survivors, S2 and S3, neutralized MARV plaque formation by at least 50%, our pre-determined threshold (figure 4b). Uninfected control serum samples had very low neutralization values, illustrating the specificity of this response in MARV survivors.

We evaluated serological responses longitudinally with serum samples collected every 6 months. For our purposes, we used a threshold of ≥50% neutralization of MARV virus (PRNT$_{50}$) to determine positive neutralization responses. For samples collected 9 and 15 months after the outbreak, 2/6 survivors had neutralizing antibody titers between 1:20 and 1:40 (figure 3b and table 2). Neutralizing responses to MARV began to diminish 21mo. after the outbreak and dropped below our threshold after 27mo. In contrast, antibody titers to irradiated MARV over this same timeframe remained consistent with no drop in antibody end titer (table 2).

Whereas our current knowledge of T cell responses to various filovirus infections suggests common themes, such as robust Th1-skewed CD4 T cell responses, the antibody responses appear to be more divergent. MARV survivors generate IgG responses against GP, NP (all survivors), VP40 (S2-7), VP30 (S1-4,6,7) but not to VP35 or VP24 (table 1). These profiles of viral protein reactivity resemble the serological profile of SUDV survivors (Sobarzo et al., 2013). Whereas the reactivity against viral proteins may be similar, the neutralizing antibody response in MARV survivors appears to differ greatly from that seen in SUDV survivors. Analyses of survivors of the SUDV outbreak in Gulu, Uganda demonstrate long-lived neutralizing antibody titers (Sobarzo et al., 2013).   Admittedly, this particular MARV cohort is small in number; however, in a similar sample size for a recent SUDV outbreak in Kibaale, Uganda (Albarino et al., 2013), 5/5 survivors had neutralizing serum responses (Sobarzo et al., 2015). Even more striking is the magnitude of neutralizing titers among recent SUDV survivors: 3/5 had PRNT$_{50}$ values at or above 1/80. Neutralizing antibody responses in a MARV survivor have been previously reported (Flyak et al., 2015) but our data is the first longitudinal analysis to demonstrate a decline in these responses, despite maintaining high antibody titers overall. Our first serum samples were obtained ~9 mo. after the outbreak so it remains possible that all survivors may have had neutralizing responses at time points more proximal to infection. What is clear, however, is that these responses are lower in magnitude than analogous SUDV survivors (Sobarzo et al., 2015).

Neutralizing antibody responses have been achieved through vaccination against MARV GP in mouse, guinea pig and cynomolgus macaques (Grant-Klein et al., 2015; Shedlock et al., 2013), though the duration of the neutralizing response was not determined. Whereas neutralizing antibodies elicited by vaccination against filoviruses is a coveted immune response, functions of non-neutralizing antibodies have been described in other viral immune responses. Non-neutralizing antibodies to HIV and LCMV glycoproteins inhibit infection of DCs and macrophages (Holl et al., 2006) and limit virus spread (Hangartner et al., 2006), respectively. Various non-neutralizing functions of

antibodies elicited by vaccination against HIV have been described in great detail (Chung et al., 2014; Chung et al., 2015). Perhaps more intriguing is a report that non-neutralizing antibodies elicited by vaccination against influenza NP can play a role in aiding the T cell response in protecting mice against influenza infection (Carragher et al., 2008). Indeed, immunization with influenza-NP antibody complexes elicited IFNγ production from CD8 and CD4 T cells (Zheng et al., 2007), indicating a Th1-skewed immune response. As all the MARV survivors in this have antibodies recognizing MARV NP (table 1), these studies describing a role for NP-specific antibodies in T cell responses may provide a roadmap for the ontogeny of the MARV survivor immune responses described herein.

These studies are the first to provide a detailed longitudinal analysis of immune responses among human MARV survivors. Our findings highlight that while T cell responses may be common among human filovirus survivors, the neutralizing antibody response varies to a greater degree. The discord in neutralizing responses between MARV and SUDV survivors indicate that there is a critical knowledge gap regarding what can be considered a protective response to filovirus infection.

Materials and Methods

Study design
      Subjects included confirmed survivors, according to patient PCR and ELISA results, from the MARV outbreak of 2012 in the Ibanda and Kabale districts of Uganda, as well as healthy local community members that were not infected.

Ethics statement
      The study was approved by the Helsinki committees of the Uganda Virus Research Institute in Entebbe, Uganda (reference number GC/127/13/01/15), Soroka Hospital, Beer-sheva, Israel (protocol number 0263-13-SOR) and the Ugandan National Council for Science and Technology (UNCST) (registration number HS1332). Written informed consent as well as a personal health questionnaire was completed for each subject.

Flow cytometry assays
      Whole blood cultures were established as previously reported (Sobarzo et al., 2016). PBMCs were collected in CPT vacutainers (BD Biosciences) and isolated according to the manufacturer's protocol. Total cell yields were split between various culture conditions in RPMI+5%FBS: no stimulation, 50 μg recombinant MARV or SUDV GP, 10 μg irradiated MARV or SUDV, or SEB (1 μg). Culture volume across all conditions was 1mL. After 2hrs, cultures were supplemented with monensin and antibodies against CD40L and CD107a. Total culture time was 18hrs. Following stimulation, cells were stained with the amine-reactive Aqua dye (Thermo Fisher) to detect dead cells, nonspecific staining was blocked with 1% mouse serum, and surface proteins stained with fluorescently-labeled antibodies. Following fixation and permeabilization, intracellular cytokines were detected. Samples were acquired on an LSRII (BD Biosciences) at the MRC/UVRI facilities in Entebbe, Uganda. Flowjo (version X, Treestar) was used to analyze flow cytometry data.

Plaque reduction neutralization test
      Plaque reduction neutralization tests (PRNT) were performed as previously described (Sobarzo et al., 2016). Neutralization titers were determined to be the last dilution of serum that reduced the number of plaques by 50% compared with control wells. Plaque reduction neutralization assays were performed in the BSL-4 lab of USAMRIID (Fort Detrick, Frederick, Maryland, USA).

Cytokine and chemokines detection using Q-Plex™ ELISA-based chemiluminescent assay
      Levels of human cytokines were detected using Q-Plex technology (Quansys Biosciences, Logan, Utah, USA) according to the manufacturer's instructions. Readouts were obtained with a Quansys Imager (Quansys Biosciences) and results analyzed using the Q-View Software program (Quansys Biosciences).

ELISA antigens
      For ELISA assays, irradiated MARV (Ci67 isolate), recombinant MARV GP$_{1-649}$, and total 293T cell lysate that expressed a given recombinant MARV protein (NP, VP24, and VP35) were used as the capture antigens. Total IgG was detected with an anti-human IgG antibody conjugated to HRP. ABTS (KPL, Gaithersburg, MD) was used as

the substrate for irradiated MARV end titer ELISAs and a chemiluminescent substrate was used in ELISAs for viral proteins.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software 6.01 (GraphPad Software, Inc. LA Jolla, CA, USA). Correlation analysis was assessed by the Spearman nonparametric test. Differences in cytokine values between study groups were assessed by analysis of variants (ANOVA) and Wilcoxon rank sum test; p-values represent 2-sided p values, and p values < 0.05 were considered statistically significant.

Author Contributions

Experiments were designed by JJL, LL, JMD and SWS. SWS, ASH, AIK, AS, PB, CA, JJL, LL, and JMD acquired and processed blood samples. SWS, ASH and ME performed flow cytometry experiments under the supervision of SC. AIK, RMJ, PB, and CA performed serological assays. Supernatant cytokines were measured by AS. Data analysis was performed by SWS (flow cytometry, ELISA, cytokines), RMJ (ELISA), AIK (PRNT), AS (cytokines) and PB and CA analyzed lysate ELISAs.

Acknowledgements

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Figure 1. Analysis of MARV survivor T cell responses in whole blood cultures. Heparinized whole blood samples were collected approximately 9 months after the 2012 MARV outbreak in Uganda. A) Representative IFNg and IL-2 responses in CD4 and CD8 T cells to recombinant SUDV GP or MARV GP, irradiated SUDV or MARV, and SEB for 22hrs are shown. B) The frequency of IFNγ single-positive, IL-2 single-positive, or IFNγ and IL-2 double-positive responses among total CD4 T cells are shown for six MARV survivors.

Figure 2. Multiplex ELISA for secreted cytokines. Supernatant was collected after 22hr stimulation of whole blood cultures. Average values for the indicated cytokine secretion are reported among survivor and control populations. * indicates $p<0.05$ for irradiated MARV vs irradiated SUDV stimulations. # indicates $p<0.05$ for irradiated MARV stimulation vs resting cultures. n.s. indicates no significant difference.

Figure 3. Flow cytometry analysis of MARV survivor CD4 T cell responses in PBMC cultures. Purified PBMCs collected 27mo after the MARV outbreak were stimulated with antigens as before for a total of 18hrs. Monensin, CD40L antibody, and CD107a antibody were added after 2hrs. A) CD40L and IFNγ staining on CD4 T cells after stimulation with irradiated MARV antigen. Upper panels depict responses seen in survivors and uninfected control responses are depicted in the middle panel. The lower panel demonstrates IFNγ and TNFα staining after gating on CD40L+ CD4+ T cells from survivors as identified in the upper panel. B) Bar graph shows the frequency of total cytokine+ CD40L+ CD4+ T cells after stimulation with the indicated MARV and SUDV antigens. C) Pie charts display the composition of the CD40L+ cytokine response in MARV survivors after stimulation with irradiated MARV.

Figure 4. MARV survivor CD8 T cell responses. PBMCs were cultured as before. Plots depict IFNγ+ and CD107a+ CD8 T cell responses after stimulation with irradiated MARV. Left panels depict staining of uninfected control samples. Right panels show representative staining of four MARV survivors with and without apparent CD8 T cell responses. Positive CD8 T cell responses are considered to be CD107a+ IFNγ+.

Figure 5. Antibody responses from MARV survivors. A) Irradiated MARV was coated on plates to capture MARV-specific antibodies from serum samples. Total IgG was detected using an anti-human IgG-HRP antibody and ABTS substrate. End titer is reported as the antilog of the reciprocal of the last dilution of serum that exceeds a threshold based on naïve serum. The limit of detection is depicted by the dashed line. B) Beginning at 1:10, serial 1:2 dilutions of serum samples from MARV survivors and uninfected controls were incubated with MARV prior to inoculation of Vero E6 cells. The percentage of neutralization is reported at 1:10, 1:20, 1:40, and 1:80 dilutions based on the reduction of plaques relative to control MARV-infected Vero cells. Dashed line indicates 50% neutralization, or $PRNT_{50}$, which is used to define positive neutralizing responses.

**Table 1**. **MARV survivor reactivity to individual viral proteins**.

Table 2. Longitudinal analysis of antibody response. Antibody end titer and $PRNT_{50}$ titers are shown at 6 month intervals beginning 9 months after the end of the 2012 MARV outbreak in Uganda. n.d. indicates that a value was not determined due to sample inavailability.

Figure 1

A
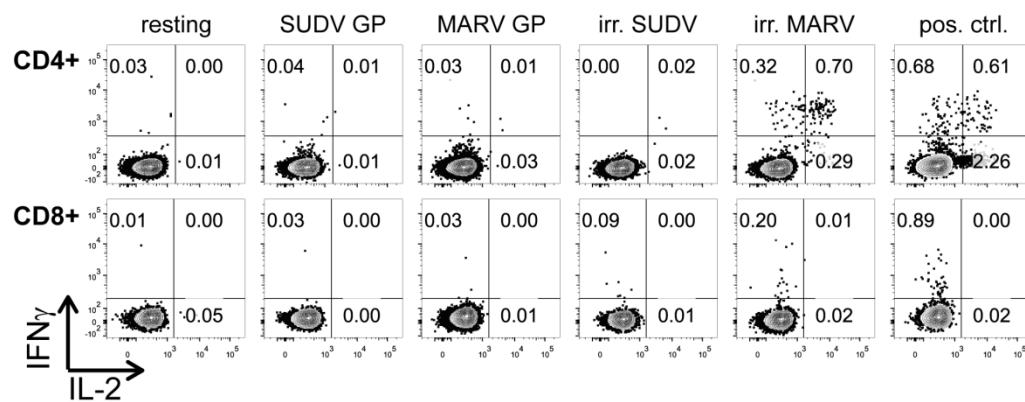
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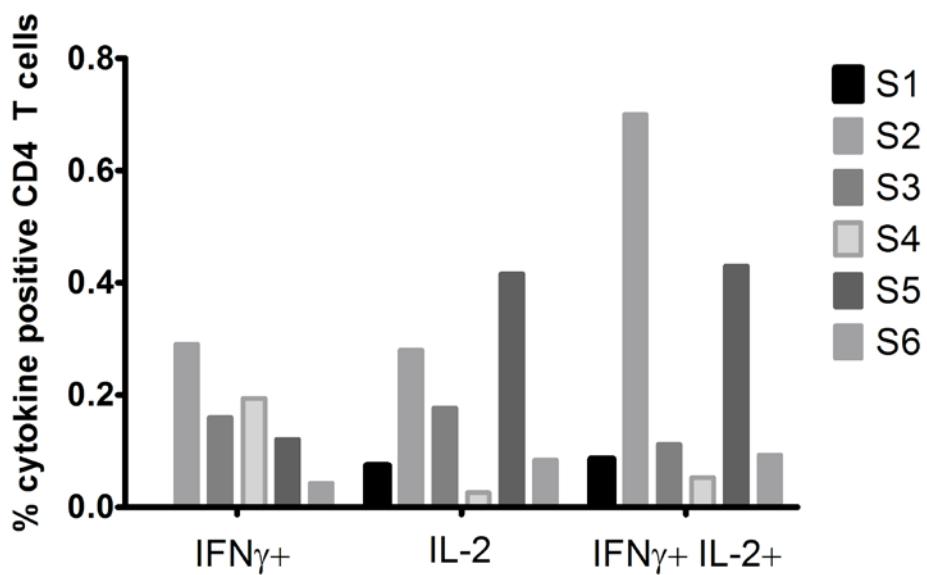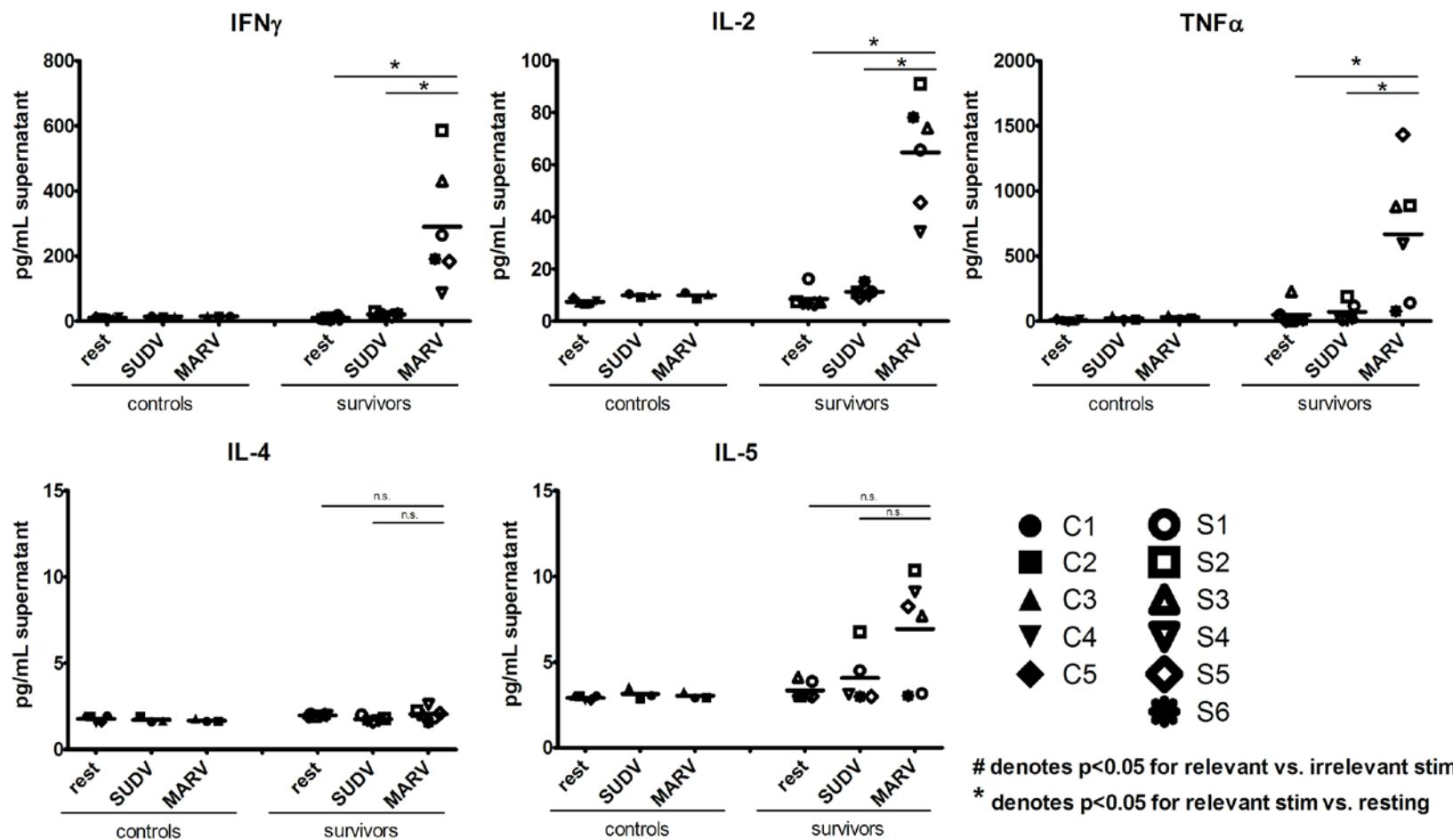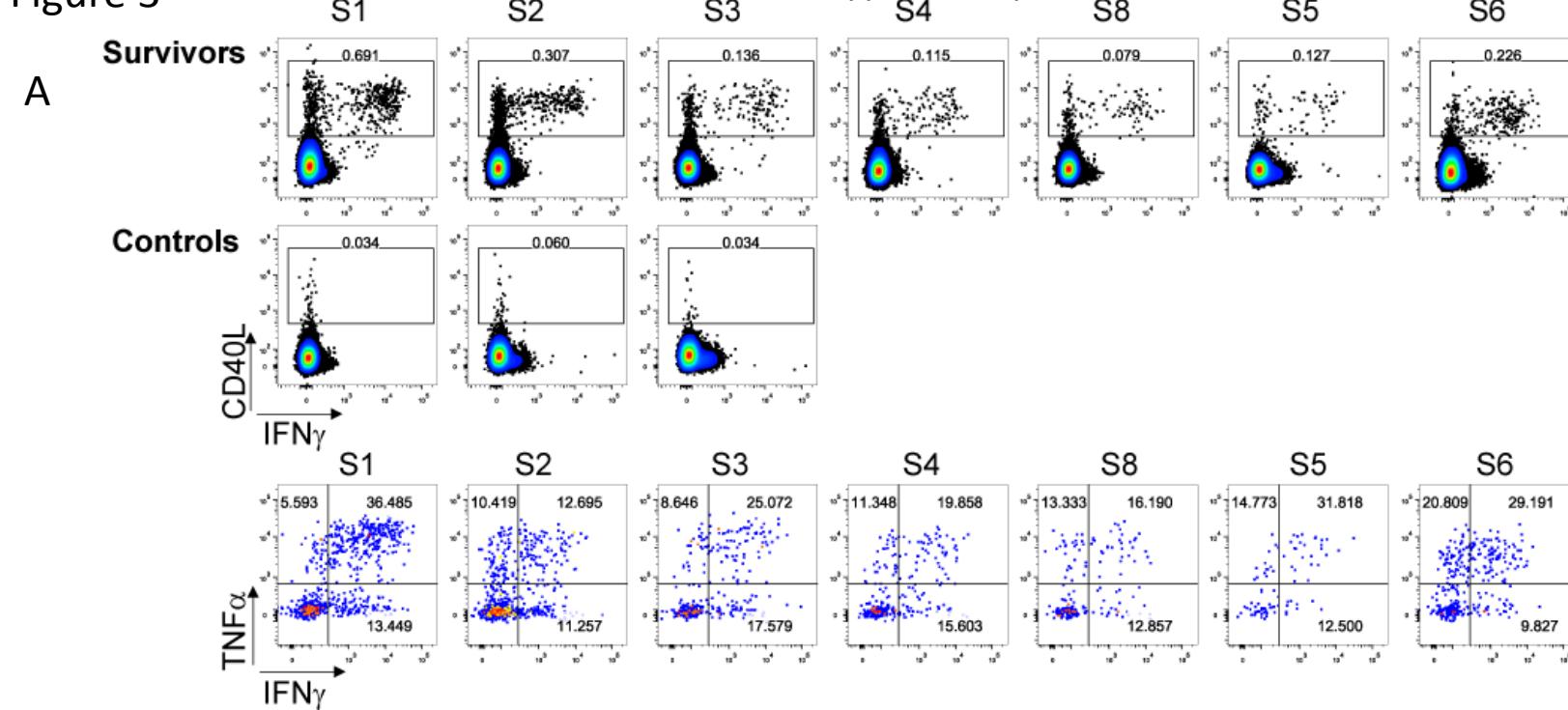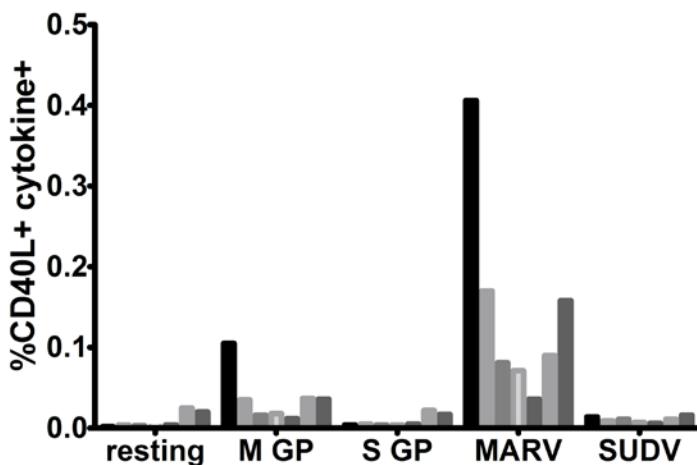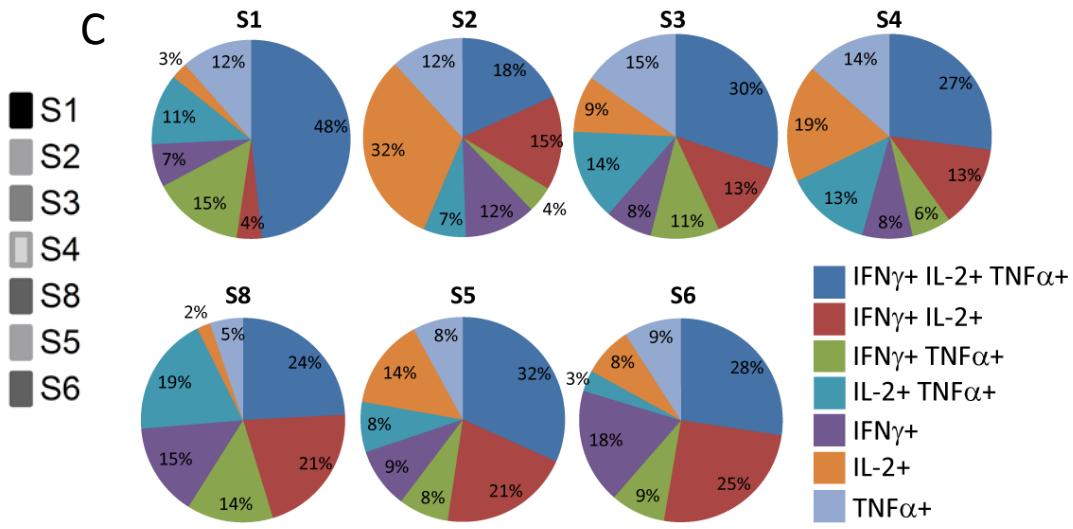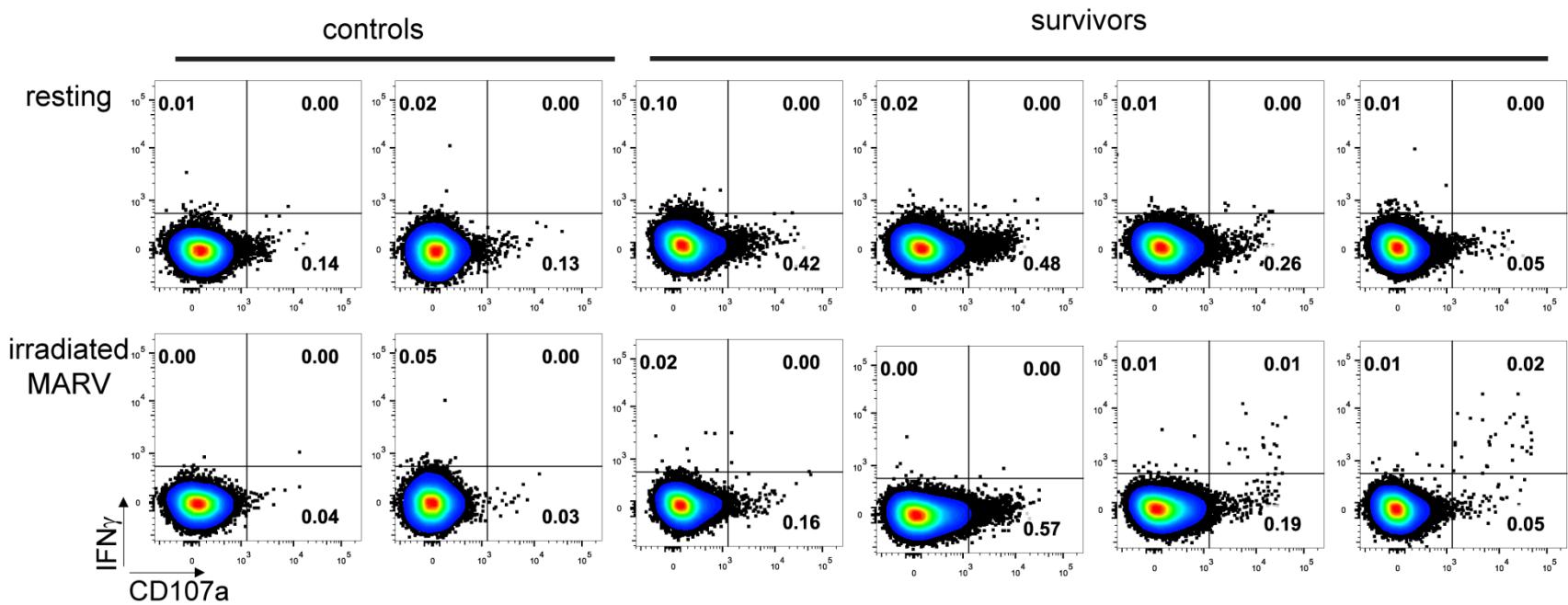
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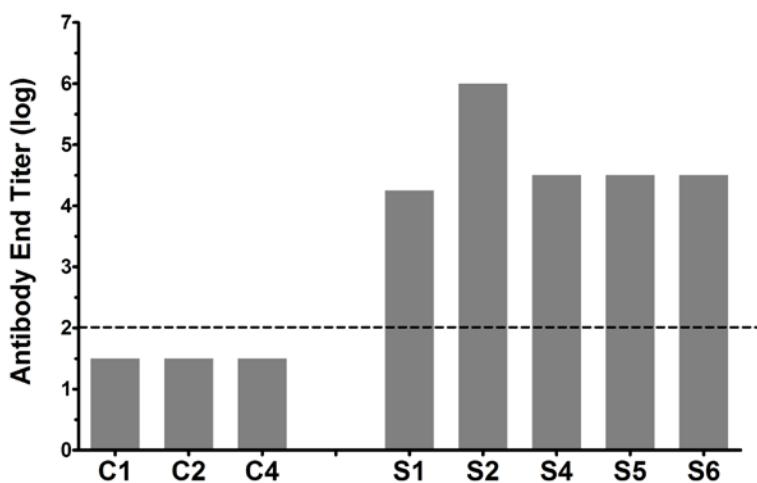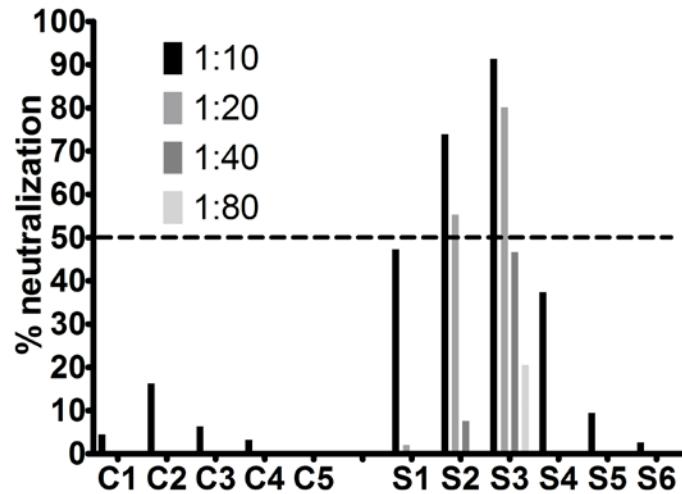
	GP	NP	VP35	VP24	VP40	VP30	Whole virus
S1	+	+	-	-	-	+	+
S2	+	+	-	-	+	+	+
S3	+	+	-	-	+	+	+
S4	+	+	-	-	+	+	+
S5	+	+	-	-	-	-	+
S6	+	+	-	-	+	+	+
S7	+	+	-	-	+	+	+
S8	+	+	-	-	+	-	+

Table 2

		Time of collection post-outbreak				
		9 mo.	15 mo.	21 mo.	27 mo.	33 mo.
S1	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.25	4	4	3.75	4
S2	$PRNT_{50}$	20	20	10	<10	<10
	End titer	6	5.75	5.5	5.5	6
S3	$PRNT_{50}$	40	40	n.d.	<10	<10
	End titer	5.5	5.0	5.5	6	5.5
S4	$PRNT_{50}$	<10	10	<10	<10	<10
	End titer	4.5	4.5	4.25	4.0	4.5
S5	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.5	4	4.5	4.5	4.5
S6	$PRNT_{50}$	<10	<10	<10	<10	<10
	End titer	4.5	4.5	4.75	5.5	6
S7	$PRNT_{50}$	n.d.	10	n.d.	n.d.	n.d.
	End titer	n.d.	4.5	n.d.	n.d.	n.d.
S8	$PRNT_{50}$	n.d.	<10	<10	<10	<10
	End titer	n.d.	4.5	n.d.	4.75	4.75